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Detection of Murine Typhus Infection in Fleas by Using the Polymerase Chain Reaction

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Polymerase chain reaction (PCR) amplification of DNA was used to detect the etiologic agent of murine typhus, *Rickettsia typhi*, in experimentally infected adult fleas. A primer pair derived from the 17-kilodalton antigen sequence of typhus and spotted fever group rickettsiae was used to amplify a 434-base-pair (bp) fragment of the genome of the murine typhus rickettsiae. The amplified 17-kilodalton protein antigen-specific sequence was detected in ethidium bromide-stained agarose gels in individual fleas as early as 2 days after exposure to rickettsemic rats (two of six tested). The 434-bp sequence was not detected in uninfected control fleas. A dot hybridization assay used to detect the 434-bp fragment was also specific and about 100-fold more sensitive than the agarose gel PCR assay. Since the PCR assay employed a boiled extract of triturated fleas, both PCR and an antigen capture enzyme-linked immunosorbent assay (ELISA) could be performed on the same individual flea homogenate. The ELISA identified 12 infected fleas out of 29 randomly selected fleas, compared with 14 specimens which were positive by PCR. The PCR assay detected rickettsiae in samples in which no viable rickettsiae were detected by plaque assay. Like the ELISA, the PCR assay sensitivity was due in part to its suitability for detecting small numbers of both live and dead *R. typhi* in fleas.

The pathogenic rickettsiae are a group of intracellular bacteria responsible for a variety of human diseases. These diseases include Rocky Mountain spotted fever (*Rickettsia rickettsii*), epidemic typhus (*Rickettsia prowazekii*), murine typhus (*Rickettsia typhi*), scrub typhus (*Rickettsia tsutsugamushi*), boutonneuse fever (*Rickettsia conorii*), and rickettsial pox (*Rickettsia akari*) (13). Since these rickettsiae are transmitted by a wide range of arthropods (e.g., ticks, fleas, lice, and mites), their detection in the vector arthropods is of major importance for epidemiological studies and control strategies. A number of detection techniques have been developed, including direct or indirect immunofluorescence, enzyme-linked immunosorbent assays (ELISAs) with polyclonal or monoclonal antibodies, and recovery of the agents from vectors by culture in embryonated eggs or tissue culture cells or by experimental infections in laboratory animals (5, 6, 12). Despite the sensitivity and reliability of some of these techniques, most of them require extensive laboratory support as well as antisera to well-defined antigens. They are also time-consuming and expensive. Their field applicability has some obvious limitations. In addition, the recovery of the pathogenic organisms from infected vectors requires either live or properly frozen specimens and their delivery to the laboratory facilities. Subsequent isolation, purification, and identification of the rickettsiae from the arthropod vectors require procedures which are difficult and entail some risk of infection.

The polymerase chain reaction (PCR), which uses specific oligonucleotide primers and *Taq* DNA polymerase to synthesize copious quantities of DNA from a single template (9), provides a valuable new approach to the detection and identification of pathogenic rickettsiae within infected vectors. In particular, the recent sequencing of a rickettsial 17-kilodalton antigen which is common to members of both the spotted fever and the typhus group rickettsiae (1, 2) makes it possible to select both group-cross-reactive and group-specific primers and probes for the diagnosis of rickettsiae either in vectors, in tissue samples, or in blood from infected individuals (M. Carl, C. W. Tibbs, M. E. Dobson, S. Paparello, and G. A. Dasch, J. Infect. Dis., in press). In this report, the PCR detection of a sequence within the 17-kilodalton antigen gene of *R. typhi* in experimentally infected fleas is described. We present a comparison of the sensitivity of PCR with that of ELISA detection and direct isolation of rickettsiae from fleas. The utility of the PCR is further enhanced by development of a dot hybridization assay for the PCR product and elimination of the need for isolating purified DNA from the infected flea.

MATERIALS AND METHODS

Rickettsial strain. *R. typhi* AZ332 (Ethiopian) (3) was used to infect fleas. Infected *Xenopsylla cheopis* fleas were obtained after feeding them on rickettsemic laboratory rats as described previously (6). Flea samples were collected at day 0 and every other day postinfection (d.p.i.) thereafter and stored in tubes containing 0.5 ml of brain heart infusion broth (BHI) at −70°C.

Diagnosis by PCR. (i) Selection of sequences for primers and probes. A pair of oligonucleotide primers (primer 1 GCTCT TGCAACTTCTATGTT and primer 2 CATTGTTCGTCAG GTTGGCG) was synthesized on the basis of the DNA sequence (published data) of the gene encoding the 17-kilodalton protein antigen from *R. rickettsii* (1). Each of the two 20-base oligomer primers was complementary to a region of DNA in which *R. rickettsii*, *R. conorii*, *R. prowazekii*, and *R. typhi* have very similar nucleotide sequences (2) (base 10 of primer 1 is G for *R. typhi* and *R. prowazekii*; bases 6, 9, and 18 of primer 2 are C, A, and A, respectively, for *R. prowazekii*); therefore, this pair of primers could amplify *R. typhi* DNA. The length of the

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rickettsial genome targeted for amplification was predicted to be 434 base pairs (bp) (Carl et al., in press). The specificity of these primers was tested in PCR, using purified DNA from various bacteria. A single band with the length predicted for amplified DNA was obtained with *R. rickettsii*, *R. prowazekii*, *R. typhi*, and *R. canada* McKiel but not with *Ehrlichia risticii* ATCC VR-986, *Escherichia coli* K-12, *Wolbachia persica* ATCC-VR 331, *Rochalimaea quintana* Fuller, and *R. tsutsugamushi* strains Karp, Kato, and Gilliam (Carl et al., in press).

(ii) Preparation of samples for PCR analysis. Individual fleas (infected and uninfected) were triturated in 100 μl of BHI and boiled for 10 min. The PCR was carried out, using 10 μl of the boiled suspension. Serial 10-fold dilutions of a yolk sac seed AZ332 strain of *R. typhi* were used to establish the sensitivity of the PCR. This seed had the absolute rickettsial body count of 9.24×10^9/ml and 6.07×10^8 tissue culture PFU/ml (3). Control DNA was prepared from purified *R. typhi* by proteinase K-sodium dodecyl sulfate digestion and then repeated phenol and chloroform extractions and ethanol precipitation. For PCR studies, we also utilized frozen aliquots of specimens which were used in our previous studies (5, 6). As a result, the information regarding the rickettsial titers in terms of PFU, direct immunofluorescent antibody test (DFA), or ELISA was available for some of the materials. In brief, the quantitation of rickettsiae in infected fleas was carried out by using the chicken embryo cell plaque assay method (6) on either individual fleas or the homogenates of pooled fleas (n = 10 to 20 per time point). The presence of rickettsiae in individual fleas was determined by DFA, using fluorescein isothiocyanate-labeled anti-*R. typhi* (convalescent) guinea pig serum (6). Double-sandwich antigen capture ELISA was performed on either individual or pooled flea samples as described previously (5). In addition to these samples, other flea specimens were also prepared and tested simultaneously by both ELISA and PCR.

(iii) Amplification. PCR was run as described previously (Carl et al., in press) on a Thermal Cycler (Perkin-Elmer Cetus, Norwalk, Conn.). Each 10-μl sample was amplified for 35 repeated cycles of denaturation at 94°C for 30 s, annealed at 57°C for 2 min, and subjected to the sequence extension step at 70°C for 2 min in the presence of 2 U of *Taq* polymerase (Perkin-Elmer Cetus) and each of the four deoxynucleotide triphosphates in the reaction mixture (100 μl total).

(iv) Detection and identification of *R. typhi* sequence in reaction products. The *R. typhi* target DNA sequence amplified by PCR was identified by visualization of a 434-bp band on agarose gels after electrophoresis and dot blot hybridization. In the agarose gel method, 14 μl of the PCR product was subjected to electrophoresis in 1% gels (SeaKem; FMC Bioproducts, Rockland, Maine), and the gels were stained with ethidium bromide and examined for bands of appropriate size with UV transillumination. In the dot blot hybridization method, PCR products (10 μl) were combined with 5 μl of 3 M NaOH and 40 μl of TE (10 mM Tris [pH 8.0]–1 mM EDTA [pH 8.0]) and incubated for 60 min at 65°C. The pH was neutralized by the addition of 50 μl of 2 M ammonium acetate. The samples were applied to nylon membrane (Schleicher & Schuell, Inc., Keene, N.H.), using a filtration manifold. The filters were dried and prehybridized at 45°C for 24 h in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–4× Denhardt solution–100 μg of denatured and sonicated herring sperm DNA per ml. PCR-amplified *R. typhi* DNA was labeled with ^{32}P by using a random primer DNA labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and hybridized to the membrane at 45°C for 16 h in the prehybridization solution. The blot was washed first in 1× SSC–0.1% sodium dodecyl sulfate for 30 min at room temperature; the membrane was transferred to 0.1× SSC–0.1% sodium dodecyl sulfate for 1 h at 65°C. The membrane was dried and exposed to Kodak XAR film (Eastman Kodak Co., Rochester, N.Y.) with an intensifying screen.

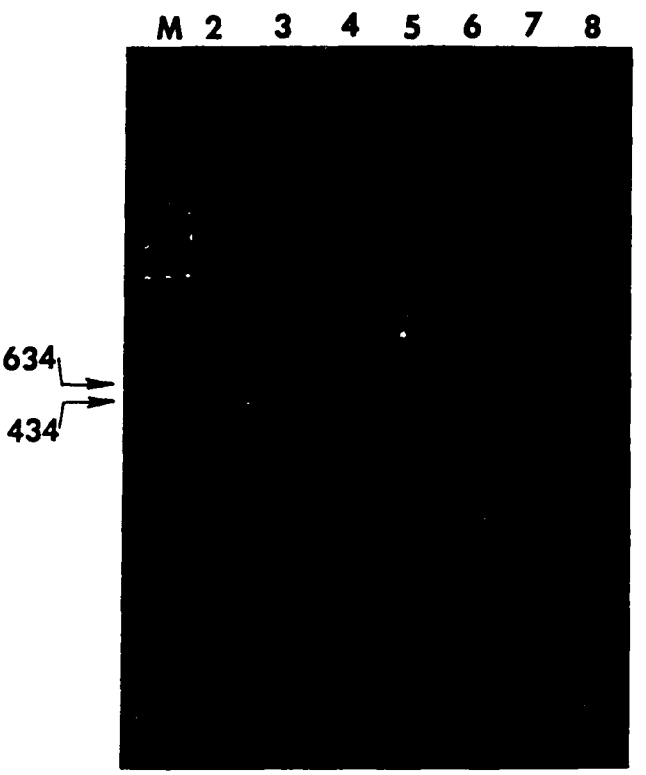

FIG. 1. Agarose gel analysis of PCR products of boiled serial 10-fold dilutions of BHI seeded with *R. typhi* (Ethiopian strain). Each lane represents the number of PFU per milliliter of BHI: 10^5 through 10^0 rickettsiae (lanes 2 through 7, respectively). M, High-range DNA size standards (100 to 23,000 bp), Bio-Rad Laboratories, Richmond, Calif. Lane 8, BHI.

RESULTS

Sensitivity and specificity of PCR and dot hybridization detection. Our initial studies revealed that extraction of purified DNA from either fleas or rickettsial seeds was not necessary in the PCR assay. For example, DNA extracted from 10^7 PFU of rickettsiae produced a faint PCR band in agarose gel (data not shown) when compared with the same rickettsiae which had been boiled for 10 min prior to PCR assay and used without further processing. Consequently, this simplified sample treatment was employed prior to PCR amplification in all subsequent experiments.

To standardize PCR and to evaluate the sensitivity and specificity of the primer pair, 10^7 PFU of *R. typhi* seed was added to 1 ml of BHI. A 10-μl portion from each 10-fold serial dilution was then boiled and PCR amplified for 35 cycles. Agarose gel electrophoresis of the PCR products revealed the predicted band of 434 bp for samples containing 10^5 through 10^2 rickettsial PFU/ml (Fig. 1, lanes 2 to 5) but not lower dilutions (Fig. 1, lanes 6 and 7). Even greater sensitivity was obtained when dot hybridization was used to

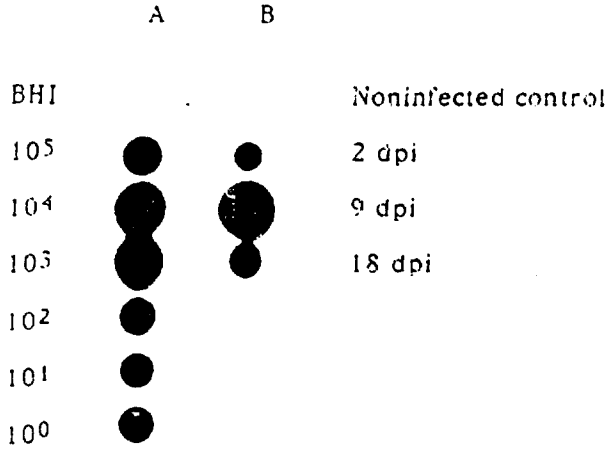

FIG. 2. Dot hybridization of PCR products of *R. typhi* (Ethiopian strain)-seeded BHI (A). The amplification products from sensitivity studies shown in Fig. 1 were dot blotted onto a nylon membrane and probed with ^{32}P-end-labeled *R. typhi* DNA probe. Selected amplified products from whole uninfected and infected fleas at different days after infectious feeding (dpi) were dot blotted in the same manner (B). Subsequent dot blot analysis of decimal dilutions of the seed (10^2 to 10^{-2}) was performed to determine the endpoint titer. Dot blot failed to detect rickettsial DNA in the amplified PCR products of the seed at the dilutions of 10^{-1} to 10^{-2} *R. typhi* PFU/ml.

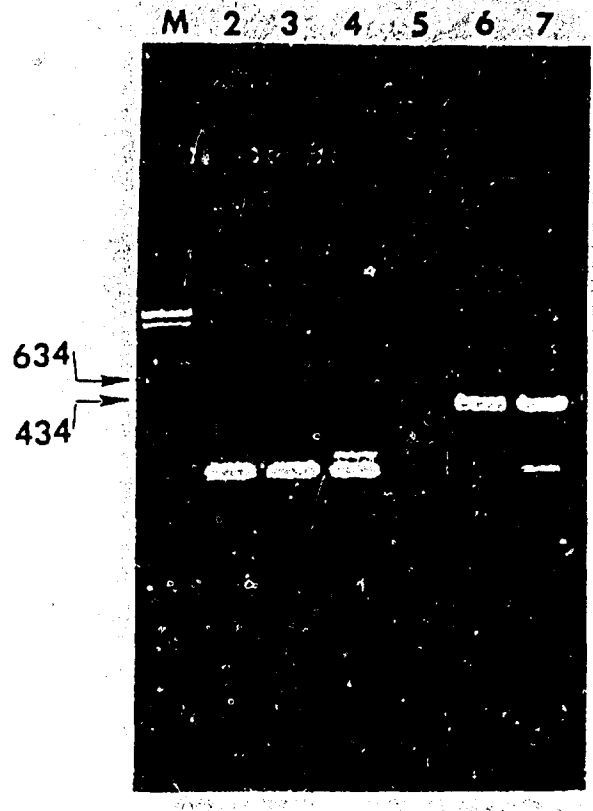

FIG. 3. Agarose gel electrophoresis of PCR-amplified products from uninfected fleas (lanes 2 to 4), 10^2 *R. typhi*-seeded BHI (lane 6), and a flea at 9 d.p.i. (lane 7). M, Low-range DNA size standards (42 to 1,746 bp, Bio-Rad). Lane 5, Blank.

assay amplification products, since even 1 rickettsial PFU/ml of the reaction mixture could be detected (Fig. 2). The reaction products from uninfected individual flea specimens did not contain the detectable 434-bp band in agarose gel (Fig. 3, lanes 2 to 4), thus excluding amplification of PCR product from the endogenous microflora and endosymbionts of the flea. Known infected samples including BHI seeded with 10^0 *R. typhi* PFU (Fig. 3, lane 6) and fleas at 9 d.p.i. (Fig. 3, lane 7) revealed bands of the expected size. The reaction products of uninfected fleas which were seeded with 10^4 PFU of rickettsiae also produced the predicted 434-bp band (data not shown).

Correlation of PCR detection of *R. typhi* in fleas with other methods. Infected fleas from different batches with known PFU per flea or ELISA titers were used in the PCR reaction. None of the PCR samples of uninfected fleas revealed a band of the expected size (Fig. 3 to 5). Two out of three fleas at 2 d.p.i. (Fig. 4, lanes 4 to 6), a single flea at 9 d.p.i. (Fig. 4, lane 8), and four out of four fleas at 18 d.p.i. (Fig. 5, lanes 2 to 5) were positive for *R. typhi* by PCR. Detection of infection in fleas at 2 d.p.i. (67%) is a good example of the sensitivity of the PCR method. We were not successful in detecting *R. typhi* infection in either fleas at 2 d.p.i. or uninfected individual fleas (n = 20) as tested by DFA or in the pooled (20 to 50 fleas per pool) samples of fleas at 1 to 3 d.p.i. by tissue culture plaque assay. In addition, the optical reading in single flea ELISA for fleas at 2 d.p.i. only revealed one out of six as positive (optical density, 0.141 for the positive flea). Most flea samples (n = 18) of 9, 10, 14, 18, and 23 d.p.i. which were positive by DFA and ELISA were also positive by PCR (Table 1). Increases in the intensity of the 434-bp band in samples at 9 to 18 d.p.i. correlated with rickettsial loads in those fleas. The PFU per flea as measured by tissue culture plaque assay ranges from 10^4 to 10^7 for fleas at ~9 d.p.i. (Table 1) (6). Dot blot hybridization on selected PCR-positive flea samples further confirmed the results obtained by gel electrophoresis (Fig. 2).

TABLE 1. *R. typhi* detection in experimentally infected *X. cheopis* fleas: gel assay PCR versus double-sandwich ELISA

Type of flea[a]	No. of fleas positive/total by:		Rickettsial titer ($\log_{10}$ PFU per flea (range))
	ELISA	PCR	
Uninfected	0/10	0/10	<1
At 2 d.p.i.	1/6	2/6	<1
At 9–10 d.p.i.	8/12	7/12	6.2 (5.4–7.6)
At 18 d.p.i.	1/6	4/6	6.5 (6.4–6.6)
At 23 d.p.i.	2/5	1/5	

[a] Fleas were washed, surface sterilized, and homogenized in 100 μl of BHI. This preparation was then boiled for 10 min and used in either PCR (10 μl) or ELISA (50 μl).

[b] Flea samples were collected from different experiments to include fleas with both high and low infection rates.

DISCUSSION

Murine typhus occurs in many areas of the world and is more common than is generally realized (4). Murine typhus is a household infection because of its intimate association with commensal rats and their fleas. Nearly all human cases have been associated with commensal rats and their fleas, in particular *X. cheopis* fleas (11). Since *X. cheopis* fleas are the key factor in the transmission cycle of murine typhus to humans in many endemic areas, field surveys to evaluate the prevalence of *R. typhi* infection in these fleas should be conducted from time to time. Such evaluation would generate important epidemiological information and provide a baseline for effective control programs. Currently available field survey methodology is cumbersome. The application of

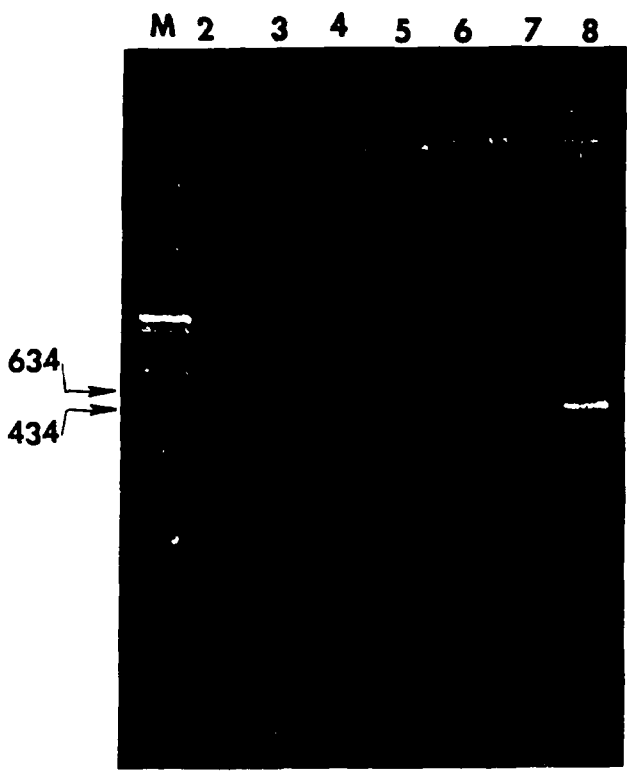

FIG. 4. Agarose gel analysis of PCR products of an uninfected flea (lane 3), fleas at 2 d.p.i. (lanes 4 to 6), and a flea at 9 d.p.i. (lane 8).

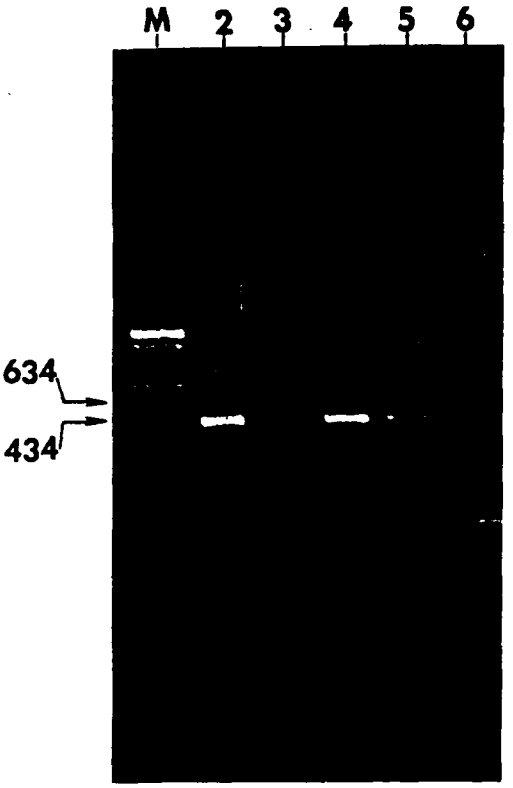

FIG. 5. Agarose gel analysis of PCR products of fleas at 18 d.p.i. (lanes 2 to 5) and an uninfected flea (lane 6).

PCR to detect *R. typhi* infection in vector fleas provides several advantages over other well-established, sensitive, and specific assays namely ELISA and DFA. Unlike ELISA, DFA, and plaque assays, PCR requires no fresh or properly frozen specimens. PCR has been applied by others to fixed tissues (frozen or Formalin fixed) (10), museum specimens (8), and even alcohol-preserved specimens. Such versatility of PCR makes it very advantageous in field studies by reducing the potential dangers involved in the maintenance and transportation of the infected vectors. For example, insecticides can be applied to collect fleas, and such fleas could be directly processed for PCR reaction.

In addition to facilitating the collection of fleas for control purposes under field conditions, PCR detection of *R. typhi* appears to be significantly more sensitive than the ELISA, DFA, or plaquing techniques. Even though only one-fifth as much sample was employed in PCR as that employed in ELISA, the simple agarose gel visualization of PCR product was as good as or better than ELISA for detecting *R. typhi* in DFA or plaque assay-negative fleas in the first few days after feeding on rickettsemic rats. As demonstrated here, the dot assay was 100-fold more sensitive than the agarose gel visualization technique. Consequently, portions of samples negative by agarose gel analysis could be screened by dot assay to ensure that poorly infected fleas were not missed.

Since the dot assay was positive with 10 μl of a dilution of 1 PFU/ml it appears that assay sensitivity correlated better to total rickettsial count than to PFU (15:1, rickettsial body to uncentrifuged PFU). Even centrifugation only increases plaque counts four- to sixfold so that the PCR dot assay appears to detect both live and dead rickettsiae in the samples. Although the number of genomic copies per rickettsia is not known precisely, the PCR dot assay endpoint appears to correspond remarkably closely to one rickettsial body. To further confirm the presence of *R. typhi* rather than *R. prowazekii* or spotted fever rickettsiae in the fleas, only primer pairs specific for *R. typhi* are being evaluated. This development will be most important in confirming the presence of *R. typhi* in mixed ectoparasite pools or in potential new vectors. Alternatively, the same assay used here may be suitable for detecting *R. prowazekii* in both lice and fleas or *R. rickettsii* in ticks.

The agarose gel assay and dot PCR assays for *R. typhi* are both semiquantitative. Although a quantitative PCR assay is not needed for field studies, further refinements in the current method by densitometric quantitation of product, serial dilutions of sample, or an alternative solution hybridization protocol (7) will permit replacement of animal titrations or difficult plaque assays for refined laboratory studies (3, 5, 12). Indeed, the sensitivity of PCR may permit studies of transovarial transmission in ectoparasites or quantitation of persistence of rickettsiae in animals which presently cannot be done in any other way.

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